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Original Article

DESIGN AND CREATION OF VAGINAL LACTIC ACID BACTERIA ASSOCIATION RESISTANT TO PHYSIOLOGICAL CYCLIC PH DISCHARGES

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ABSTRACT

Aim: The aim of this study is creation of sustainable consortium of symbiotic probiotic bacteria with high colonization potential for rapid response to vaginal acute and chronic challenges.

Material and method: In this study used biocompatible lactobacilli and cocci possessing high antimicrobial activity, isolated previously from vaginal samples of healthy women from 20 to 45 years old from Armenia.

The following methods: screening of antimicrobial activity, microbial adhesion to solvents, auto aggregation and co aggregation assays were used Statistical analysis used: Student's test computer, taking the criterion of P <0.05 sufficient for significant differences in the results.

Results: The symbiotic consortium of Lactobacillus acidophilus GH 201 and Lactococcus lactis GH 204 strains shows higher inhibitory activities against bacterial pathogens and Candida albicans, intensive growth rate and biomass accumulation, than the single cultures The consortium is sustainable during multiple sub culturing imitating up and down changes of vaginal pH.

Conclusion: The sustainable consortium of symbiotic probiotic bacteria have high colonization potential for rapid response to vaginal cyclic pH discharges caused by menses and frequently intercourses. These bacteria possess enhances growth rate, antimicrobial and adhesive effects, when working together.

KEYWORDS: *LABS Consortium, Antimicrobial Activity, Adhesion, Auto aggregation, Co Aggregation*

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INTRODUCTION

Lactic acid bacteria (LAB) are considered the dominant microflora of healthy woman's vagina, they produce lactic acid lowering vaginal pH < 4.5, hydrogen peroxide (H_2O_2), bacteriocins and other antibacterial substances that protect from sexually transmitted infectious [1-3]. Various factors; nutrition, hygiene, stress, infections, low immunity, etc., destroyed vaginal microbial balance, which dramatically reducing lactobacilli and brings to propagation of resident anaerobe microorganisms, which cause bacterial vaginosis [4]. The vaginal ecosystem is sensitive to disturbances caused by menses and human activities such as frequently intercourses, douching, using of antibiotics for non-vaginal illnesses and other habits and practices. Over the course of the menstrual cycle, vaginal levels of hormones and glycogen vary, and menstrual blood (pH 7.32) alters vaginal pH

and provides a substrate for many microorganisms. Nevertheless, levels of vaginal lactobacilli appear to remain constant throughout the cycle; non-Lactobacillus species increase during the proliferative phase, while *Candida albicans* concentrations are highest towards menstruation [5].

The buffer capacity of the vagina concedes the buffer capacity of ejaculate (40 mM/pH) after sexual intercourse [6]. The alkaline buffering action of the ejaculate (~ pH 7.6) liquidates vaginal acidity for some hours after sexual intercourse and reacidification rate of vagina is 0.5 pH units/h [7].

Lactobacilli colonization efficiency depends on their adaptive (propagation rate, epithelial cells adhesion, stability towards stress) and probiotic (intracellular interaction, antimicrobial compounds synthesis; lactic acid, hydrogen peroxide, bacteriocins) properties, as well as immunomodulation abilities [8, 9]. For enhancing of colonization, efficiency currently used multistrain probiotic preparations, which usually consist of non-symbiotic lactobacilli belonging different species, which often partially or fully disappear after a few menses and/or frequent sexual intercourses. It is expected that LABs symbiotic consortium of lactococci and lactobacilli will be more beneficial for vaginal colonization/recolonization, because the lactococci are growth well at elevate pH and by reducing of it will promote the growth of lactobacilli, adapted to low pH of vagina.

The aim of this study is creation of sustainable consortium of symbiotic probiotic bacteria with high colonization potential for rapid response to vaginal acute and chronic challenges.

MATERIAL AND METHODS

Culture Media

LAPTg (yeast extract, peptone, tryptone, glucose, Tween 80) broth, M16 (Merck, BRG), Saburo agar (Himedia, India), Nutrient triptose agar (Ferak, Berlin).

Enzymes: proteinase K, pepsin, trypsin (Himedia, India).

Solvent: xylene (Merck, BRG)

Microorganisms

LABs: Laboratory collection isolated and identified earlier.

The test microorganisms: *Escherichia coli* MDC 5003, *Staphylococcus aureus* MDC 5233, *Candida albicans* MDC 8013 (Microbial Depositary Center of "Armbiotechnology" SPC, NAS of Armenia.

Culture Growth

In test tubes bacteria were grown overnight under anaerobic conditions, then aliquots were inoculated in Erlenmeyer flasks, which containing 20 ml MRS broth and grows at 37°C in aerobic condition with agitation up to late-stationary phase. The cultures` OD was measured at λ= 600 nm by spectrophotometer.

Screening of Antimicrobial Activity

The supernatant liquids effects of 40 vaginal LABs on the growth of pathogens were studied by using of plate-diffusion method [10]. Triptose agar plates (standardized volume, 15 ml) were prepared with 10^6–10^7 CFU of each pathogen. Neutralized supernatants of LABs were placed with discs (diameter, 5 mm) in the pathogen-inoculated plates. After 5 h incubation at room temperature the plates were incubated at 37 °C for 24 h. As a positive result was defined a

clear inhibition zone of > 7 mm diameter.

The Sensitivity of Antagonistic Substances of Supernatant Fluids to Proteases

Solutions for each enzyme (pepsin, trypsin and proteinase K) in concentration 1 mg/ml are prepare previously. The 1 ml of culture`s supernatant filled into the tube and added 0.1 ml of each enzyme solution left one minute at room temperature then disk impregnated with this solution and placed on a test-culture widespread growth. Plates stored at 10^0C for 4 hours and placed in the thermostat at 37^0C. After 24 hours, the inhibitory zones are measured and compared with the results of no treated supernatants.

Hydrogen Peroxide Production by Lactobacilli

Hydrogen peroxide production by lactobacilli will measured by Peroxide test strips (Merck, Germany)

Biocompatibility Test

For this test on LAPTg agar surface were dropped 10 µl of overnight cultures giving 3 mm diameter spot, dried then at a distance of 1-2 mm spotted the second culture for partially overlap of the first spot, dried and plates incubated for 24 hours at 37°C. The cultures thought biocompatible if full merger of spot borders was remarked.

Microbial Adhesion to Solvents

Microbial adhesion to solvents (MATS) was estimated by the method of Rosenberg et al. (1980) with modifications [11]. In the stationary phase bacteria were harvested by centrifugation for 15 min at 5000 g, then washed twice and resuspended to approximately 10^8 CFU/ml in 0.1 M KNO_3 (pH 6.2). The cell suspension absorbance was measured at 600 nm (A_0). 3 ml of cell suspension was added in 1.0 ml of solvent. The two phase system was mixed by vortexing for 2 min after 10 min preincubation at room temperature. Then after 20 min of incubation at room temperature the aqueous phase was removed, and its absorbance (A_1) was measured at 600 nm. The percentage of microbial adhesion to solvent was computed by this formula: $(1-A_1/A_0) \times 100$.

Coaggregation Survey

Microorganisms were grown in LAPTg broth at 37^0C for 18 h. The cells were harvested by centrifugation for 15 min at 5000 g, then washed twice and resuspended to give viable counts of approximately 10^8 CFU/ml in phosphate buffered saline (PBS) 2 ml of each cell suspension were mixed together for 10 s by vortexing. Control tubes containing 4 ml of each microbial suspension on its own. The absorbance of the suspensions (A) was measured at 600 nm after mixing and after 5 h of incubation at room temperature. The coaggregation percentage was calculated by using of Handley et al. equation [12]:

$$Coaggregation\ (\%) = \frac{\frac{(Ax + Ay)}{2} - A(x+y)}{\frac{Ax + Ay}{2}} x\ 100,$$

Where x and y represent each control tube strains, and (x + y) the mixture.

Autoaggregation Assay: Bacterial cells in LAPTg broth were harvested by centrifugation for 5 min at 3000 rotation per minute (rpm), then washed and resuspended in PBS to give an OD = 1 at 600 nm (10^9 CFU/ml). For study of autoaggregation suspension of LAB (4 mL) mixed by vortexing. Absorbance of cells was measured immediately, after 5 h

and 24 h. Autoaggregation percentage was calculated by using of this equation –

$$(1 - \frac{At}{Ao}) \times 100,$$

Where A_t represents absorbance at different times (t= 5 h or 24 h) and A_0 represents absorbance at the beginning of the study (0 h) [13].

Statistical Analysis: Statistical analysis of the data was performed using Student's test computer, taking the criterion of $P < 0.05$ sufficient for significant differences in the results.

RESULTS AND DISCUSSIONS

Antimicrobial Activity and Biocompatibility of Vaginal Labs

In this study from 25 vaginal lactobacilli and 15 lactococci supernatant fluids possessing high antimicrobial activity against *S. aureus*, *E. coli*, *C. albicans* and on base of biocompatibility test results the *L. lactis* GH 204 and *Lactobacillus acidophilus* GH 201 pair was created for farther investigation. The antimicrobial activity of grown together overnight culture supernatant of *L. lactis* GH 204 and *L. acidophilus* GH 201 is much powerful, than mono cultures. *L. lactis* GH 204 and *L. acidophilus* GH 201 culture supernatants made inhibitory zones mean 15 and 25 mm, respectively, when in the case of mixed culture antimicrobial activity was 25-35 mm.

The Sensitivity of Antimicrobial Substances of Supernatant Fluids to Proteases

For definition of antagonistic feature of supernatant fluids of *L. lactis* GH 204 and *L. acidophilus* GH 201 cultures the supernatants were treated by proteases (proteinase K, pepsin and trypsin) and antimicrobial activity was tested by disc diffusion method on *E. coli* MDC 5003, *C. albicans* MDC 8013 and *S. aureus* MDC 5233 lawns. The results are presented in **Table 1**.

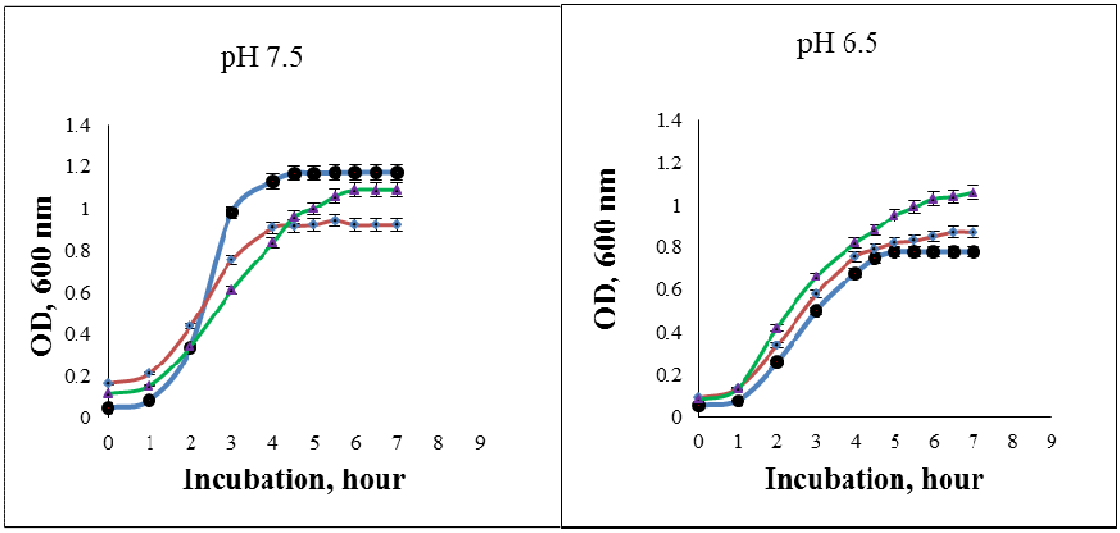
Table 1: Protease Influences on Antimicrobial Activity of Lactobacilli Supernatant Fluids

LAB Strain	Test-Strains Inhibitory Zones Around Disks Impregnated in Intact and Treated by Proteases Supernatants, Mm					
	E. coli		*C. albicans*		*S. aureus*	
	Intact	Treated	Intact	Treated	Intact	Treated
L. lactis GH 204	15 ± 0.3	8 ± 0.3	14 ± 0.3	8 ± 0.3	12 ± 0.3	8 ± 0.3
L. acidophilus GH 201	25 ± 0.3	15 ± 0.3	26 ± 0.3	15 ± 0.3	18 ± 0.3	12 ± 0.3

As shown in Table 1, *L. acidophilus* GH 201 supernatant fluid antimicrobial activity after protease treating mainly retained. It means that the antimicrobial activity of this strain is not caused by protein like compounds. The hydrogen peroxide test enabled to reveal high amount (up to 30 mg/l) H_2O_2 in supernatant of strain *L. acidophilus* GH 201. *L. lactis* GH 204 supernatant fluid antimicrobial activity after protease treating significantly reduced, which indicates on protein feature of antagonistic substances. The rest activity may be caused by undefined low molecular compounds. The hydrogen peroxide was not obtained.

The Cultural Characteristic of the Consortium and Single Strains

The growth rate of the cultures was investigated at initial pH 7.5 and 6.5 of LAPTg at 37^0C. The pH 7.5 is beneficial for growth of lactococci and pH 6.5 for lactobacilli. OD of cultures were checked every 30 min. The dates are presented in **Figure 1**.



A) B)

Figure 1: The growth Rate of the Cultures Under pH 7.5 and 6.5 (●- *L. Lactis* GH 204, ◊- *L. Acidophilus* GH 201, Δ- Consortium)

As seen in the Figure 1, at pH 7.5 *L. lactis* GH 204 growth faster, than *L. acidophilus* GH 201, whereas the consortium of the cultures starting growth at rate of lactobacillus, but then overcome it in accumulation of biomass. At pH 6.5, as it was predicted the consortium rate of growth is significantly higher, than the single cultures.

Adhesion, Auto Aggregation and Co Aggregation Properties of Vaginal Labs

The study of bacterial adhesion *in vivo* is a difficult procedure, for this reason developed the *in vitro* model system for estimation of adhesive potential of microorganisms [14-18]. Adhesion facilitated by bacterial cell surface hydrophobicity. The MATS is *in vitro* method, which used to evaluate the hydrophobic/hydrophilic cell surface properties. The strains *L. acidophilus* GH 201 and *L. lactis* GH 204 cells surface hydrophobicity was evaluated by use of apolar solvent xylene (**Figure 2**).

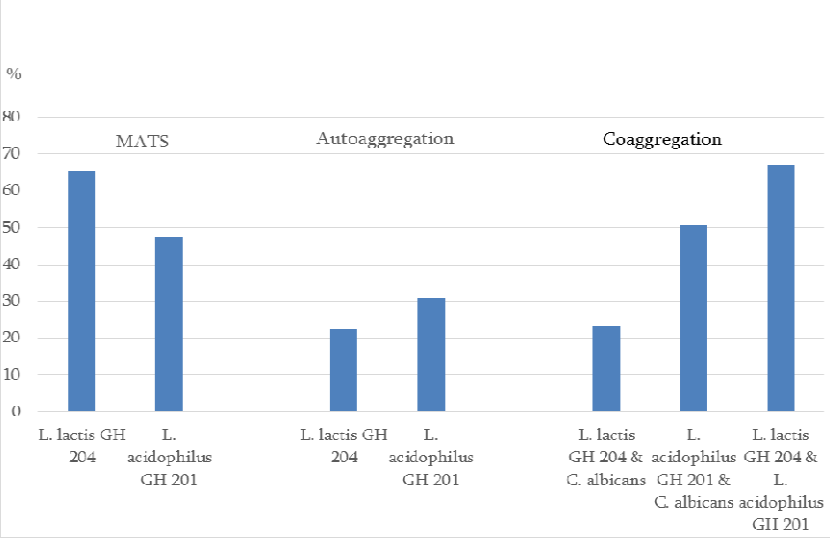


Figure 2: Adhesion, Auto Aggregation and Co Aggregation Properties of Vaginal Labs

The results indicated that the both strains, particularly *L. lactis* GH 204 showed strong affinity for xylene. The adhesion property of *L. lactis* GH 204 is significantly higher in comparison to known strains of *L. lactis*, isolated from human origin [19].

The strains also demostrated high autoaggregation properties, particularly lactococci, which important for their adhesion to epithelial cells [14, 20]

The coaggregation rate of lactobacteria indicates on their colonization potential. The high grade coaggregation of *L. acidophilus* GH 201 with *L. lactis* GH 204 could increase colonization potential and sustainability of their consortium. The results from Figure 2, demonstrated high coaggregation level (67.3 %) between the lactobacilli and lactococci, which is very important for vaginal colonization.

On the other hand, LABs coaggregation with pathogen microorganisms increase antimicrobial efficiency due to their target action. As seen in figure 2, coaggregation of *L. acidophilus* GH 201 and *L. lactis* GH 204 with the main causative agent of vaginosis *C. albicans* is very high, respectively 50.73% and 23.18%.

Sustainability of the Microbial Consortium

The sustainability of the association was evaluated through serial subculturing in LAPTg broth with initial pH 7.6, which reduced up to pH 4.0 in process of bacterial growth, this imitates pH changes caused by menses and sexual intercourses. The ratio of bacilli and cocci was estimated by plating on selective M16 agar over a period of 10 days and counting of small and large colonies. *L. acidophilus* GH 201 and *L. lactis* GH 204 cultures equal ratio stay constant during 10 regular subculturings.

CONCLUSIONS

The consortium of *L. acidophilus* GH 201 and *L. lactis* GH 204 strains has higher inhibitory activity than the strains themselves against bacteria associated with urogenital infectious and *C. albicans*. The mixed culture`s high growth rate can ensure rapid recovery of vaginal acidic pH discharges caused by menses or sexual intercourses. The consortium is stable during serial subculturing. The high level of autoaggregation and coaggregation increase colonization potential of mixed culture and hindered adhesion of *C. albicans* to epithelial cells, furthermore, destroy him by target action.

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